WO0052141

Publication Title:

CELLS RESISTANT TO TOXIC GENES AND USES THEREOF

Abstract:

The present invention relates to cells and cell strains that are resistant to the killing effects of one or more toxic genes, particularly those that kill hosts in the absence of a suppressing function, e.g. kicB or ccdB. The host cells may comprise one or more suppression mutations, such as deletional or insertional mutations in gyrA, endA or recA, or combinations thereof (particularly gyrA/endA or gyrA/recA), which allow cell strains carrying the one or more suppression mutations to survive the presence and/or expression of one or more toxic genes within their genome or in extrachromosomal genetic elements within the host cell. Preferred host cell strains include prokaryotic host cells, particularly specified strains of E. coli containing the gyrA462 mutation and/or one or more additional mutations, such as DB3, DB3.1, DB4 and DB5. The host cells of the invention are useful in producing recombinant genetic constructs, particularly cDNAs and cDNA libraries, via traditional genetic engineering techniques or via recombinational cloning using engineered recombination sites. The host cells are also useful in cloning and propagation of toxic genes that act upon DNA gyrase, such as ccdB.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 1/21, 15/66

A1

(11) International Publication Number: WO 00/52141

(43) International Publication Date: 8 September 2000 (08.09.00)

(21) International Application Number: PCT/US00/05246

(22) International Filing Date: 2 March 2000 (02.03.00)

(30) Priority Data:
60/122,392 2 March 1999 (02.03.99) US

(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US]; 9800 Medical Center Drive, Rockville, MD 20850 (US).

(72) Inventors: HARTLEY, James, L.; 7409 Hillside Drive, Frederick, MD 21702 (US). BRASCH, Michael, A.; 20931 Sunnyacres Road, Gaithersburg, MD 20882 (US). TEMPLE, Gary, F.; 114 Ridge Road, Washington Grove, MD 20880 (US).

(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox, P.L.L.C., Suite 600, 1100 New York Avenue, Washington, DC 20005-3934 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CELLS RESISTANT TO TOXIC GENES AND USES THEREOF

(57) Abstract

The present invention relates to cells and cell strains that are resistant to the killing effects of one or more toxic genes, particularly those that kill hosts in the absence of a suppressing function, e.g. *kic*B or *ccd*B. The host cells may comprise one or more suppression mutations, such as deletional or insertional mutations in *gyr*A, *end*A or *rec*A, or combinations thereof (particularly *gyr*A/*end*A or *gyr*A/*rec*A), which allow cell strains carrying the one or more suppression mutations to survive the presence and/or expression of one or more toxic genes within their genome or in extrachromosomal genetic elements within the host cell. Preferred host cell strains include prokaryotic host cells, particularly specified strains of *E. coli* containing the gyrA462 mutation and/or one or more additional mutations, such as DB3, DB3.1, DB4 and DB5. The host cells of the invention are useful in producing recombinant genetic constructs, particularly cDNAs and cDNA libraries, via traditional genetic engineering techniques or via recombinational cloning using engineered recombination sites. The host cells are also useful in cloning and propagation of toxic genes that act upon DNA gyrase, such as *ccd*B.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Cells Resistant to Toxic Genes and Uses Thereof

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to cell and molecular biology. More particularly, the present invention relates to mutant host cell strains that are resistant to the effects of the expression of one or more toxic genes. Most particularly, the invention relates to such host cell strains carrying one or more mutations in their DNA gyrase gene which renders the host cell strains resistant to the effects of toxic genes that act upon DNA gyrase. The host cell strains of the invention are useful for a variety of purposes, including but not limited to amplification and cloning of nucleic acid molecules by recombinational cloning methods, and for cloning and propagation of toxic genes.

Related Art

Toxic Genes

The genes ccdA and ccdB are the antidote and toxin genes respectively of the *E. coli* F plasmid (P. Bernard, et al., *J. Mol. Biol. 234*: 534 (1993)). Together, they ensure the death of daughter cells that do not receive a copy of F. Expression of the ccdB protein interferes with the rejoining step of DNA gyrase, causing the host cell chromosome to be cut to pieces. Plasmids that contain the ccdB gene without the antidote gene can be propagated in a gyrase mutant host cell strain, such as *E. coli* gyrA462 (T. Mike, et al., *J. Mol. Biol. 225*: 39 (1992)). Other toxic genes have also been identified, for example ΦX *E* which is toxic when expressed in *E. coli* unless the host cell lacks an *sly*D gene which encodes *cis-trans* peptidyl-prolyl isomerase upon which the ΦX *E* gene acts (Liu, Q. *et al.*, *Curr. Biol. 8*:1300-1309 (1998)).

For certain applications, such as cloning of nucleic acid molecules by recombinational cloning techniques like those described herein, and for cloning

and propagation of genetic constructs containing one or more toxic genes, it would be advantageous to have a choice of mutant host cell strains that are resistant to the effects of toxic genes such as *ccd*B which is used in preferred recombinational cloning methods. The present invention provides such mutant host cell strains.

SUMMARY OF THE INVENTION

The present invention relates generally to mutant host cells and host cell strains that are resistant to the effects of the expression of one or more toxic genes. Most particularly, the invention relates to such host cells and host cell strains carrying one or more mutations, particularly in their DNA gyrase gene, which renders the host cells and host cell strains resistant to the effects of the expression of one or more toxic genes that act upon DNA gyrase. The invention also relates to host cells and host cell strains having one or more mutations which allow the host cell to grow in the presence of a toxic gene selected from the group consisting of *ccd*B, *kic*B, *Dpn*I, ΦX *E*, and the like.

Thus, in one aspect the invention provides mutant host cells, which may be *Escherichia coli* cells, containing a *gyr*A gene, an *end*A gene, and a *rec*A gene, wherein the *gyr*A and *end*A genes contain one or more mutations that render the host cell resistant to the expression of one or more toxic genes including, but not limited to, toxic genes such as *ccd*B, *kic*B, *Dpn*I, an apoptosis-related gene, a retroviral gene, a defensin, a bacteriophage lytic gene, an antibiotic sensitivity gene, an antimicrobial sensitivity gene, a plasmid killer gene, and a eukaryotic transcriptional vector gene that produces a gene product toxic to bacteria, and most particularly *ccd*B. The invention also provides such mutant host cells which further comprise one or more mutations in the *rec*A gene (including, but not limited to, Δ(*srl-rec*A)1398), and/or one or more genetic elements (including, but not limited to, a tetracycline resistance gene or transposon *Tn*10). The invention also relates to such mutant host cells which comprise one or more additional mutations, such as mutations in *rec*A, *end*A, *mcr*A, *mcr*B, *mcr*C, *hsd*, *deo*R, and the like, preferably in *rec*A or *end*A or more preferably in both *rec*A and *end*A.

Preferred mutations in the *gyr*A gene according to this aspect of the invention include gyrA462. While the invention relates to any mutant host cell or host cell strain having the features and characteristics described herein, preferred such host cells and host cell strains include, but are not limited to, a DB3 cell (deposit number NRRL B-30097), a DB3.1 cell (deposit number NRRL B-30098), a DB4 cell (deposit number NRRL B-30106), and a DB5 cell (deposit number NRRL B-30107), or a mutant or derivative thereof.

The invention also relates to host cell strains containing a mutation in the DNA gyrase gene (such as those described herein) and further containing one or more additional mutations in one or more genes selected from the group consisting of *rec*A, *end*A, *mcr*A, *mcr*B, *mcr*C, *hsd*, *deo*R, and the like, preferably in *rec*A or *end*A or more preferably in both *rec*A and *end*A. Such host cell strains are useful in cloning one or more nucleic acid molecules (*e.g.*, one or more genes) of interest, and host cell strains containing mutations in at least two genes that make them resistant to the activities of two or more toxic genes are useful, for example, in cloning two or more genes, for example by recombinational cloning methods in which the two nucleic acid molecules of interest are contained on one or more genetic constructs (*e.g.* a vector) that has two toxic genes, such that the host cell must be resistant to both toxic genes in order to grow and express (or replicate) the two or more genes of interest.

In another aspect, the invention relates to methods of cloning a genetic construct comprising one or more toxic genes, such as those toxic genes described above. Methods according to this aspect of the invention preferably comprise introducing a genetic construct comprising one or more toxic genes into one or more of the host cells or host cell strains of the invention, and cultivating the host cell or host cell strain under conditions favoring the clonal expansion of the host cell.

In another aspect, the invention relates to kits comprising one or more of the mutant host cells or mutant host cell strains of the invention. Kits according to this aspect of the invention may comprise one or more of the host cells or host cell strains of the invention, and may further comprise one or more additional components suitable for use with, or for cultivation of, the host cells or host cell

strains of the invention. Such additional components may include, for example, one or more culture media suitable for cultivation of the host cells or host cell strains of the invention, one or more selection agents (such as one or more antibiotics, dyes, detergents, antimicrobial agents, and the like), one or more genetic constructs comprising one or more toxic genes (such as a vector comprising one or more of the toxic genes described herein, most preferably *ccd*B), one or more buffers, and the like.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of what is known in the art, in light of the following drawings and description of the invention, and in light of the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depiction of an Entry Vector, containing the *ccd*B toxin gene flanked by recombination sites attL1 and attL2, and a kanamycin resistance (Kan^r) gene.

Figure 2 is a schematic depiction of a Destination Vector, containing the *ccd*B toxin gene and an inactive *ccd*A antidote gene, flanked by recombination sites attR1 and attR2, and an ampicillin resistance (amp^r) gene.

Figure 3 is a depiction of the cloning sites of the Entry Vector pENTR-7, showing the location of the *ccd*B gene in relation to the flanking attL1 and attL2 recombination sites and the multiple cloning sites contained in this vector.

Figure 4 is a restriction map of the Destination Vector pTrc-DEST1, showing the location of the *ccd*A and *ccd*B genes in relation to the flanking attR1 and attR2 recombination sites, the ampicillin resistance gene, and the multiple cloning sites contained in this vector.

Figure 5 is a schematic depiction of recombinational cloning, using vectors carrying the *ccd*B gene.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In the description that follows, a number of terms used in molecular and cellular biology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Host cell: A "host" or "cell" as these terms are used herein, and which terms may be used interchangeably with each other and with the terms "host cell" and "host cell strain," includes prokaryotic or eukaryotic organisms that can be genetically engineered. Typical prokaryotic host cells that may be used in accordance with the present invention include, but are not limited to, bacterial cells such as those of the genera *Escherichia* spp. (particularly *E. coli*), *Streptomyces* spp., *Erwinia* spp., *Klebsiella* spp., *Bacillus* spp. (particularly *B. cereus*, *B. subtilis*, and *B. megaterium*), *Serratia* spp., *Pseudomonas* spp. (particularly *P. aeruginosa*) and *Salmonella* spp. (particularly *S. typhi* or *S. typhimurium*). It will be understood, of course, that there are many suitable strains and serotypes of each of the host cell species described herein, any and all of which may be used in accordance with the invention. Preferred as a host cell is *E. coli*, and particularly preferred are *E. coli* strains RR1 (*E. coli* F- *mcr*B *mrr* *hsd*S20(r_B- m_B-) *rec*A13 *leu*B6 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L20(Sm^r) *sup*E44 λ-), DH10B (*E. coli* F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*, *leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ-), Stbl2 (*E. coli* *mcr*A (*mcr*BC-*hsd*RMS-*mrr*)*end*A1 *rec*A1*thigyr*A96*rel*A1*sup*E44(*lac-pro*AB) λ-) DH5α, and BL21Si, which are available commercially (Life Technologies, Inc; Rockville, Maryland). Typical eukaryotic host cells that may be used in accordance with the present invention include, but are not limited to, animal cells (particularly mammalian (including human), avian, amphibian, reptilian, nematode and insect cells), plant cells, and fungal (including yeast) cells. For examples of these and other suitable hosts, *see* Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

Recombinational Cloning: is a method described herein and in U.S. Application Nos. 08/486,139, filed June 7, 1995 (now abandoned), 08/663,002, filed June 7, 1996 (now U.S. Patent No. 5,888,732), 09/005,476, filed January 12, 1998, 09/177,387, filed October 23, 1998, 09/233,492, filed January 20, 1999, 09/233,493, filed January 20, 1999, 60/122,389, filed March 2, 1999, 60/126,049, filed March 23, 1999, 60/136,744, filed May 28, 1999, 09/296,280, filed April 22, 1999, 09/296,281, filed April 22, 1999, 09/432,085, filed November 2, 1999, and 09/438,358, filed November 12, 1999, the disclosures of all of which are incorporated herein by reference in their entireties. In the recombinational cloning process, segments of nucleic acid molecules or populations of such molecules are fused, exchanged, inserted, replaced, substituted or modified, *in vitro* or *in vivo*.

Selectable marker: is a DNA segment that allows one to select for or against a molecule (*e.g.*, a replicon) or a cell that contains it, often under particular conditions. These markers can encode an activity, such as, but not limited to, production of RNA, peptide, or protein, or can provide a binding site for RNA, peptides, proteins, inorganic and organic compounds or compositions and the like. Examples of Selectable markers include but are not limited to: (1) DNA segments that encode products which provide resistance against otherwise toxic compounds (*e.g.*, antibiotics); (2) DNA segments that encode products which are otherwise lacking in the recipient cell (*e.g.*, tRNA genes, auxotrophic markers); (3) DNA segments that encode products which suppress the activity of a gene product or a functional site; (4) DNA segments that encode products which can be readily identified (*e.g.*, phenotypic markers such as β-galactosidase, green fluorescent protein (GFP), and cell surface proteins); (5) DNA segments that bind products which are otherwise detrimental to cell survival and/or function; (6) DNA segments that otherwise inhibit the activity of any of the DNA segments described in Nos. 1-5 above (*e.g.*, antisense oligonucleotides); (7) DNA segments that bind products that modify a substrate (*e.g.* restriction endonucleases); (8) DNA segments that can be used to isolate or identify a desired molecule (*e.g.* specific protein binding sites); (9) DNA segments that encode a specific nucleotide sequence which can be otherwise non-functional (*e.g.*, for PCR amplification of subpopulations of molecules); (10) DNA segments, which when absent, directly

or indirectly confer resistance or sensitivity to particular compounds; and/or (11) DNA segments that encode products which are toxic in recipient cells.

Toxic gene. A toxic gene can be a DNA that is expressed as a toxic gene product (a toxic protein or RNA), or can be toxic in and of itself. (In the latter case, the toxic gene is understood to carry its classical definition of "heritable trait".) Examples of such toxic gene products are well known in the art, and include, but are not limited to, restriction endonucleases (*e.g.*, *Dpn*I), apoptosis-related genes (e.g. ASK1 or members of the bcl-2/ced-9 family), retroviral genes including those of the human immunodeficiency virus (HIV), defensins such as NP-1, inverted repeats or paired palindromic DNA sequences, bacteriophage lytic genes such as those from ϕX174 (*e.g.*, ϕX *E*) or bacteriophage T4; antibiotic sensitivity genes such as *rps*L, antimicrobial sensitivity genes such as *phe*S, plasmid killer genes, eukaryotic transcriptional vector genes that produce a gene product toxic to bacteria, such as GATA-1, and genes that kill hosts in the absence of a suppressing function, *e.g.*, *kicB* or *ccdB*. A toxic gene can alternatively be selectable *in vitro*, *e.g.*, a restriction site.

Vector: is a nucleic acid molecule (preferably DNA) that provides a useful biological or biochemical property to an Insert. Examples include plasmids, phages, viruses, autonomously replicating sequences (ARS), centromeres, transposons, and other sequences which are able to replicate or be replicated *in vitro* or in a host cell, or to convey a desired nucleic acid segment to a desired location within a host cell. A vector can have one or more restriction endonuclease recognition sites at which the sequences can be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a nucleic acid fragment can be spliced in order to bring about its replication and cloning. Vectors can further provide primer sites, *e.g.*, for PCR, transcriptional and/or translational initiation and/or regulation sites, recombinational signals, replicons, Selectable markers, *etc*. Clearly, methods of inserting a desired nucleic acid fragment which do not require the use of homologous recombination, transpositions or restriction enzymes (such as, but not limited to, UDG cloning of PCR fragments (U.S. Patent No. 5,334,575, entirely incorporated herein by reference), T:A cloning, and the like) can also be applied

to clone a fragment into a cloning vector to be used according to the present invention. The cloning vector can further contain one or more selectable markers suitable for use in the identification of cells transformed with the cloning vector.

Other terms used in the fields of recombinant DNA technology and molecular and cell biology as used herein will be generally understood by one of ordinary skill in the applicable arts.

Host Cells

One aspect of the invention provides host cells and host cell strains that are resistant to the killing (bacteriocidal) or growth suppressive (bacteriostatic) activities of one or more toxic genes. Such host cells are useful in a variety of methods, including for example propagating nucleic acid molecules containing one or more toxic genes, and selection of host cells which have been successfully transformed with a genetic construct containing a gene of interest and a toxic gene.

A number of such selection schemes can be used with a variety of host cells, particularly *E. coli* cells and cell strains. One is to put a repressor gene on one segment of the subcloning plasmid, and a drug marker controlled by that repressor on the other segment of the same plasmid. Of course a way must exist for growing such a plasmid, *i.e.*, there must exist circumstances under which the killer gene will not kill. There are a number of these genes known which require particular strains of *E. coli*. One such scheme is to use the restriction enzyme *Dpn*I, which will not cleave unless its recognition sequence GATC is methylated. Many popular common *E. coli* strains methylate GATC sequences, but there are mutants in which cloned *Dpn*I can be expressed without harm. Other restriction enzyme genes may also be used as a toxic gene for selection. In such cases, a host containing a gene encoding the corresponding methylase provides protected hosts for use in the invention. Similarly, the *ccd*B protein is a potent poison of DNA gyrase, efficiently trapping gyrase molecules in a cleavable complex, resulting in DNA strand breakage and cell death. Mutations in the gyrA subunit of DNA gyrase, specifically the gyrA462 mutation contained in the *E. coli* RR1 gyrA462

mutant designated DB1, confers resistance to *ccd*B (Bernard and Couturier, *J. Mol. Biol. 226*: 735-745 (1992)).

Hence, in one aspect the invention relates to mutant host cells and host cell strains that are resistant to the effects of the expression of one or more toxic genes. Host cells of this aspect of the invention may comprise one or more mutations in one or more genes within their genomes or on extrachromosomal or extragenomic DNA molecules (such as plasmids, phagemids, cosmids, etc.), including mutations in, for example, *rec*A, *end*A, *mcr*A, *mcr*B, *mcr*C, *hsd*, *deo*R, and the like, preferably in *rec*A or *end*A or more preferably in both *rec*A and *end*A Most particularly, the invention relates to such host cells and host cell strains carrying one or more mutations, particularly in their DNA gyrase gene, which renders the host cells and host cell strains resistant to the effects of the expression of one or more toxic genes that act upon DNA gyrase.

In a first such aspect the invention provides mutant host cells, which may be *Escherichia coli* cells, containing a *gyr*A gene, an *end*A gene, and a *rec*A gene, wherein the *gyr*A and *end*A genes contain one or more mutations that render the host cell resistant to the expression of one or more toxic genes. According to the invention, the one or more mutations may render the host cells and host cell strains resistant to toxic genes including, but not limited to, *ccd*B, *kic*B, *Dpn*I, an apoptosis-related gene, a retroviral gene, a defensin, a bacteriophage lytic gene, an antibiotic sensitivity gene, an antimicrobial sensitivity gene, a plasmid killer gene, and a eukaryotic transcriptional vector gene that produces a gene product toxic to bacteria, and most particularly *ccd*B. The invention also provides such mutant host cells which further comprise one or more mutations in the *rec*A gene (including, but not limited to, Δ(*srl-rec*A)1398), and/or one or more genetic elements (including, but not limited to, a tetracycline resistance gene or transposon *Tn*10). Preferred mutations in the *gyr*A gene according to this aspect of the invention include gyrA462

One such host cell strain, *E. coli* strain DB2, has been constructed in accordance with the invention. DB2 cells contain the gyrA462 mutation and a mutation in endA. DB2 cells containing plasmids that express the *ccd*B gene (for example, Destination and Entry Vectors described below) are not killed by *ccd*B.

This strain is available from Life Technologies and was deposited on October 14, 1997, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-21852.

Analogous mutant host cell strains have also been produced and are provided by the invention. In particular, the invention provides additional cell strains based on the DB2 mutant strain described above. In one aspect, the invention provides strain DB3, which is based on the tetracycline resistant *E. coli* strain RR1, and which contains the *gyr*A462, *end*A and *rec*A mutations. Hence, strain DB3 may be represented as *E. coli* RR1 (*gyr*A462 *end*A (*rec*A-)). This strain (designated *E. coli* DB3) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30097.

In another aspect, the invention provides strain DB3.1, which is identical to strain DB3 (*i.e.*, it is based on *E. coli* RR1, and contains the *gyr*A462, *end*A, and *rec*A mutations) except that DB3.1 is tetracycline sensitive as it does not contain the tetracycline resistance (tet^r) gene carried by the other RR1-based strains (RR1, DB1, DB2 and DB3). Strain DB3.1 may therefore be represented as *E. coli* RR1 (*gyr*A462 *end*A (*rec*A-) tet^s). This strain (designated *E. coli* DB3.1) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30098.

Other mutant host cell strains have also been constructed and are provided by the invention. In this aspect, the mutant host cell strains are based on the tetracycline-resistant *E. coli* DH10B strain (available commercially from Life Technologies, Inc.). In one such aspect, the invention provides strain DB4, which is a DH10B *E. coli* strain that carries the *gyr*A462 mutation and a deletion in the endA gene, as well as carrying the tetracycline resistance transposon *Tn*10. Strain DB4 thus may be represented as *E. coli* DH10B (*end*A Δ(*srl*-*rec*A)1398 ::Tn*10*(tet^r)). This strain (designated *E. coli* DB4) is available from

Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30106.

In another aspect, the invention provides strain DB5, which is identical to strain DB4 (*i.e.*, it is based on *E. coli* DH10B, and contains the *gyr*A462 mutation and the deletion in the endA gene), except that DB5 is tetracycline sensitive as it does not contain the tetracycline resistance (tet^r) *Tn*10 transposon carried by DB4. Strain DB5 thus may be represented as *E. coli* DH10B (*end*A Δ(*srl-rec*A)1398). This strain (designated *E. coli* DB5) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30107.

Each of these DB mutant host cell strains (DB1, DB2, DB3, DB3.1, DB4, and DB5) are resistant to the effects of expression of the *ccd*B gene by the host cell. Other mutant host cell strains which contain one or more mutations rendering the host cells resistant to *ccd*B may also be produced and characterized by the skilled artisan in accordance with the guidance contained herein in combination with information known in the art. In addition, other mutant host cell strains resistant to other toxic genes will also be apparent to one of ordinary skill based on the teachings contained herein and the knowledge in the art, and are encompassed within the scope of the present invention. In one such aspect, these host cell strains of the invention may be mutant cell strains that are resistant to one or more alternative, or one or more additional, toxic genes, including but not limited to *kic*B, *Dpn*I and other restriction endonucleases, apoptosis-related genes (*e.g.*, ASK1 or members of the bcl-2/ced-9 family), retroviral genes including those of the human immunodeficiency virus (HIV), defensins such as NP-1, inverted repeats or paired palindromic DNA sequences, bacteriophage lytic genes such as those from φX174 (*e.g.*, φX *E*) or bacteriophage T4, antibiotic sensitivity genes such as *rps*L, antimicrobial sensitivity genes such as *phe*S, plasmid killer genes, eukaryotic transcriptional vector genes that produce a gene product toxic to bacteria such as GATA-1, and the like. Mutant host cell strains that are resistant to such toxic genes may be prepared in accordance with the guidance

herein, and may be used in methods of recombinational cloning as detailed herein and in the propagation of nucleic acid molecules or vectors containing the toxic genes which would otherwise be bacteriocidal or bacteriostatic to host cell strains not containing these particular mutations.

Production and Characterization of Mutant Host Cells

The mutant host cells and host cell strains of the invention may be produced by standard mutagenesis methods that will be familiar to one of ordinary skill in the art. For example, to generate an RR1-based mutant host cell strains of the invention (*e.g.*, DB3 and DB3.1), one may obtain an *E. coli* RR1 host cell strain (*e.g.* from Life Technologies, Inc; Rockville, MD) and mutagenize the host cells by any of a number of well-known mutagenesis methods, such as chemical mutagenesis, radiation-induced mutagenesis, and the like. Analogously, to generate a DH10B-based mutant host cell strains of the invention (*e.g.*, DB4 and DB5), one may obtain an *E. coli* DH10B host cell strain (*e.g.* from Life Technologies, Inc.; Rockville, MD) and mutagenize the host cells by any of a number of well-known mutagenesis methods, such as chemical mutagenesis, radiation-induced mutagenesis, and the like.

There are well known procedures for introducing specific mutations into nucleic acid sequences and thus for creating mutant host cell strains containing these specific mutations. A number of these are described in Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1989-1996). Mutations can also be designed into oligonucleotides, which can be used to modify existing cloned sequences, or in amplification reactions. Random mutagenesis can also be employed if appropriate selection methods are available to isolate the desired mutant DNA or RNA. Such isolation methods may include, for example, culturing the host cells in culture media (which may be solid or liquid) containing one or more selection agents (such as one or more antibiotics or antimicrobial agents, including tetracycline, ampicillin, kanamycin, chloramphenicol, and the like).

The presence of the desired mutations can be confirmed by isolating the DNA from the mutant host cell strain according to art-known methods such as

electrophoretic and chromatographic methods (*see*, *e.g.*, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1989-1996). Once the DNA has been isolated from the mutant host cells, the specific mutations present in a particular host cell strain may be determined by sequencing the DNA by well known methods, including manual sequencing methods (such as dideoxy sequencing; *see* Sanger, F., and Coulson, A.R., *J. Mol. Biol. 94*:444-448 (1975); Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA 74*:5463-5467 (1977)) or automated DNA sequencing.

To characterize a host cell or host cell strain to determine its resistance to the presence of a particular toxic gene, several approaches are available. In a first such approach, a genetic construct containing one or more toxic genes (such as those described herein, and particularly *ccd*B) may be introduced, using any of a number of chemical or physical transformation methods, into the host cells of the invention. For example, one or more Entry Vectors (Figures 1, 3) or one or more Destination Vectors (Figures 2, 4), available commercially from Life Technologies, Inc., and containing the *ccd*B gene, may be introduced into the host cell strains of the invention. The transformed host cells may then be cultivated, under conditions favoring the growth of the host cell, in culture medium which may contain one or more selection agents specific for the genetic construct containing the toxic gene. If the host cell strain is able to grow (*i.e.*, form colonies on solid medium, or increase in number or turbidity in liquid culture), the host cell is resistant to the presence of the toxic gene (*e.g.*, the *ccd*B gene in the example above where one or more Entry Vectors or one or more Destination Vectors are introduced into the host cell) and is said to be a mutant host cell strain of the invention.

In a related method, the resistance of a mutant host cell strain to the presence of one or more toxic genes may be determined by cloning a genetic construct comprising one or more toxic genes, such as those toxic genes described above and particularly *ccd*B, and subsequently examining the host cells for an increase in copy number of the genetic construct containing the one or more toxic genes. Methods according to this aspect of the invention preferably comprise introducing a genetic construct comprising one or more toxic genes into one or

more of the host cells or host cell strains of the invention, and cultivating the host cell or host cell strain under conditions favoring the clonal expansion of the host cell. Following this cultivation, DNA may be isolated as above from the host cells, and the isolated DNA analyzed for an increase in the copy number of the toxic gene.

Kits

In another embodiment, the invention relates to kits comprising one or more of the host cells of the invention. Kits according to this aspect of the invention may comprise a carrier means such as a box, carton, package, drum, or the like, which may be compartmentalized to receive in close confinement therein one or more container means such as tubes, vials, bottles, ampules, packages, envelopes, and the like. The one or more containers may contain one or more host cells of the invention. For example, a first container may contain one or more of the host cell strains of the invention, such as DB3, DB3.1, DB4, or DB5. Additional containers according to this aspect of the invention may comprise one or more components useful in accordance with the application in which the host cells or kits of the invention are to be used, for example one or more genetic constructs (for example, a plasmid, vector, phagemid, cosmid, and the like) containing one or more of the toxic genes described herein (particularly *ccd*B), one or more buffers or buffer salts, one or more detergents, one or more enzymes (such as one or more recombination proteins, *e.g.*, Int, IHF, or Xis, or combinations thereof, one or more reverse transcriptases, one or more nucleic acid polymerases, or one or more restriction enzymes), one or more nucleotides (which may be detectably labeled, as with a fluorophore, a chromophore, an enzyme, or a radioisotope), one or more proteins (such as albumin, one or more ribosomal proteins, and the like), one or more selection agents (*e.g.*, one or more antibiotics, detergents, dyes, antimicrobial agents, and the like), and/or one or more culture media or components thereof suitable for cultivation of the host cells of the invention.

Uses of Host Cells

The mutant host cells and host cell strains of the invention may be used for a variety of purposes. For example, the mutant host cells may be used to clone genetic constructs (*e.g.*, nucleic acid molecules (which may be linear or circular), vectors, plasmids, phagemids, cosmids, and the like) containing one or more of the toxic genes described herein, particularly *ccd*B. Methods according to this aspect of the invention may comprise multiple steps, for example introducing a genetic construct comprising one or more toxic genes into one or more of the host cells or host cell strains of the invention, and cultivating the host cell or host cell strain under conditions favoring the clonal expansion of the host cell. As used herein, "conditions favoring the clonal expansion of the host cell" means the optimal incubation conditions (including optimal nutritional, physical (*e.g.*, temperature, light, humidity, etc.), and chemical conditions) that provide for the most rapid and healthy growth of the host cell strain being cultivated. As a practical matter, and as one of ordinary skill will be aware, growth of a particular host cell strain may be determined by plating cultivation fluid containing the host cell onto solid culture media, incubating for an appropriate period of time, and counting colonies that develop, with a higher number of colonies indicating more optimal growth conditions. Analogously, as one of ordinary skill will also be aware, growth of a particular host cell strain may be determined by inoculating the host cell into liquid culture media, incubating for an appropriate period of time, and determining the turbidity of the culture media (*e.g.*, by spectrophotometry), with a higher turbidity indicating more optimal growth conditions. Mutant host cells of the invention will be resistant to the one or more toxic genes carried by the genetic constructs with which they have been transformed, and the genetic constructs containing the toxic genes will be replicated as the host cells grow. Hence, an increase in copy number of genetic constructs containing toxic genes may be accomplished using the host cells and host cell strains of the invention.

In another application, the host cells and host cell strains of the invention may be used in methods of recombinational cloning, whereby segments of nucleic acid molecules of interest or populations of such molecules are exchanged, fused, inserted, replaced, substituted or modified, *in vitro* or *in vivo* without the use of

restriction enzymes. Such methods of recombinational cloning are generally depicted in Figure 5, wherein an Entry Clone containing a gene of interest, flanked by *att*L1 and *att*L2 sites, is combined with a Destination Vector containing the *ccd*B gene flanked by *att*R1 and *att*R2 sites. Upon incubation, the *att*L1 and *att*R1 sites and the *att*L2 and *att*R2 sites recombine to create a functional subclone (which may be an expression vector, for example) and a by-product plasmid. Methods and applications for recombinational cloning are provided in detail in commonly owned, co-pending U.S. Application Nos. 08/486,139, filed June 7, 1995 (now abandoned), 08/663,002, filed June 7, 1996 (now U.S. Patent No. 5,888,732), 09/005,476, filed January 12, 1998, 09/177,387, filed October 23, 1998, 09/233,492, filed January 20, 1999, 09/233,493, filed January 20, 1999, 60/122,389, filed March 2, 1999, 60/126,049, filed March 23, 1999, 60/136,744, filed May 28, 1999, 09/296,280, filed April 22, 1999, 09/296,281, filed April 22, 1999, 09/432,085, filed November 2, 1999, and 09/438,358, filed November 12, 1999, the disclosures of all of which are incorporated by reference herein in their entireties.

It will be understood by one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are readily apparent and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

Examples

Example 1: Preparation and Characterization of Mutant Host Cells

The mutant host cells and host cell strains of the invention were produced by standard mutagenesis methods that will be familiar to one of ordinary skill in the art. For example, to generate an RR1-based mutant host cell strains of the

invention (*e.g.*, DB3 and DB3.1), *E. coli* RR1 host cell strains were obtained from Life Technologies, Inc. and mutagenized by chemical mutagenesis or radiation-induced mutagenesis. Analogously, to generate a DH10B-based mutant host cell strains of the invention (*e.g.*, DB4 and DB5), *E. coli* DH10B host cells were obtained from Life Technologies, Inc. and mutagenized in the same fashion as for the RR1-based host cells. Specific mutations were introduced as described in Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1989-1996).

The presence of the desired mutations was confirmed by isolating the DNA from the mutant host cell strain according to art-known methods such as electrophoretic and chromatographic methods (*see, e.g.*, Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1989-1996). Once the DNA was isolated from the mutant host cells, the specific mutations present in a particular host cell strain were determined by sequencing the DNA by well known methods (*see* Sanger, F., and Coulson, A.R., *J. Mol. Biol. 94*:444-448 (1975); Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA 74*:5463-5467 (1977)) or by automated DNA sequencing.

To characterize a host cell or host cell strain to determine its resistance to the presence of a particular toxic gene, several approaches were carried out. In a first such approach, a genetic construct containing one or more toxic genes (such as those described herein, and particularly *ccd*B) was introduced, using any of a number of chemical or physical transformation methods, into the host cells of the invention. For example, one or more Entry Vectors (Figures 1, 3) or one or more Destination Vectors (Figures 2, 4), available commercially from Life Technologies, Inc., and containing the *ccd*B gene, were introduced into the host cell strains of the invention. The transformed host cells were be cultivated, under conditions favoring the growth of the host cell, in culture medium which may contain one or more selection agents specific for the genetic construct containing the toxic gene. If the host cell strain was able to grow (*i.e.*, form colonies on solid medium, or increase in number or turbidity in liquid culture), the host cell was said to be resistant to the presence of the toxic gene (*e.g.*, the *ccd*B gene in the example above where one or more Entry Vectors or one or more Destination

Vectors are introduced into the host cell) and was said to be a mutant host cell strain of the invention.

In a related method, the resistance of a mutant host cell strain to the presence of one or more toxic genes was determined by cloning a genetic construct comprising one or more toxic genes, such as those toxic genes described above and particularly *ccd*B, and subsequently examining the host cells for an increase in copy number of the genetic construct containing the one or more toxic genes.

Example 2: Characterization of Strain DB3

RR1 *E. coli* host cells were mutagenized, and DNA isolated from the host cells, as described in Example 1 above, to generate strain DB3. Upon sequencing the isolated DNA, strain DB3 was found to contain the *gyr*A462 and *end*A mutations, and a complete deletion of the *rec*A gene. Hence, strain DB3 was represented as *E. coli* RR1 (*gyr*A462 *end*A (*rec*A-)).

This strain (designated *E. coli* DB3) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30097.

Example 3: Characterization of Strain DB3.1

DB3 *E. coli* host cells (Example 2) were mutagenized, and DNA isolated from the host cells, as described in Example 1 above, to generate strain DB3.1. Upon sequencing the isolated DNA, strain DB3.1 was found to contain the same *gyr*A462, *end*A, and *rec*A mutations as DB3, and to be tetracycline sensitive due to deletion of the tet^r gene. Hence, strain DB3.1 was represented as *E. coli* RR1 (*gyr*A462 *end*A (*rec*A-) tet^s).

This strain (designated *E. coli* DB3.1) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30098.

Example 4: Characterization of Strain DB4

DH10B *E. coli* host cells (Life Technologies, Inc.; Rockville, MD) were mutagenized, and DNA isolated from the host cells, as described in Example 1 above, and the mutated cells were transformed with the tetracycline resistance transposon *Tn*10 to generate strain DB4. Upon sequencing the isolated DNA, strain DB4 was found to contain the *gyr*A462 and *end*A mutations, a deletion at base 1398 of the *rec*A gene, and the *Tn*10 transposon. Hence, strain DB4 was represented as *E. coli* DH10B (*gyr*A462 *end*A Δ(*srl-rec*A)1398 ::*Tn*10(tet^r)).

This strain (designated *E. coli* DB4) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30106.

Example 5: Characterization of Strain DB5

DH10B *E. coli* host cells (Life Technologies, Inc.; Rockville, MD) were mutagenized, and DNA isolated from the host cells, as described in Examples 1 and 4 above, except that the mutated cells were not transformed with the tetracycline resistance transposon *Tn*10, to generate strain DB5. Upon sequencing the isolated DNA, strain DB5 was found to contain the same *gyr*A462, *end*A and *rec*A mutations as DB4, and to be tetracycline sensitive. Hence, strain DB5 was represented as *E. coli* DH10B (*gyr*A462 *end*A Δ(*srl-rec*A)1398 tet^s).

This strain (designated *E. coli* DB5) is available from Life Technologies and was deposited on February 27, 1999, with the Collection, Agricultural Research Culture Collection (NRRL), 1815 North University Street, Peoria, IL 61604 USA as deposit number NRRL B-30107.

Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or

any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 3 and 4.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution
Agricultural Research Culture Collection (NRRL)

Address of depository institution *(including postal code and country)*

1815 North University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
14 October 1997	NRRL B-21852

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Escherichia coli DB2 (RR1 gyrA462, endA)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, line 14.	
B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on an additional sheet ☒
Name of depository institution Agricultural Research Culture Collection (NRRL)	
Address of depository institution *(including postal code and country)* 1815 North University Street Peoria, Illinois 61604 United States of America	
Date of deposit 27 February 1999	Accession Number NRRL B-30097
C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*	This information is continued on an additional sheet ☐
Escherichia coli DB3	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*	
E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*	
The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*	

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 23 and 24.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution
Agricultural Research Culture Collection (NRRL)

Address of depository institution *(including postal code and country)*

1815 North University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
27 February 1999	NRRL B-30098

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Escherichia coli DB3.1

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, line 3.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☒

Name of depository institution
Agricultural Research Culture Collection (NRRL)

Address of depository institution *(including postal code and country)*

1815 North University Street
Peoria, Illinois 61604
United States of America

Date of deposit	Accession Number
27 February 1999	NRRL B-30106

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Escherichia coli DB4

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, line 12.
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐
Name of depository institution Agricultural Research Culture Collection (NRRL)
Address of depository institution *(including postal code and country)* 1815 North University Street Peoria, Illinois 61604 United States of America
Date of deposit 27 February 1999 — Accession Number NRRL B-30107
C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐
Escherichia coli DB5
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*
E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*
The indications listed below will be submitted to the international Bureau later *(specify the general nature of the indications, e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

WHAT IS CLAIMED IS:

1. A mutant host cell containing a *gyr*A gene, an *end*A gene, and a *rec*A gene, wherein said *gyr*A and *end*A genes contain one or more mutations that render said host cell resistant to the expression of one or more toxic genes.

2. The mutant host cell of claim 1, further comprising one or more mutations in said *rec*A gene.

3. The mutant host cell of claim 1, further comprising one or more mutations in one or more additional genes which render said host cell resistant to the expression of two or more toxic genes.

4. The mutant host cell of claim 1 or claim 2, further comprising one or more genetic elements that enable said mutant host cell to grow on tetracycline-containing culture media.

5. The mutant host cell strain of claim 1 or claim 2, wherein said mutation in said *gyr*A gene is gyrA462.

6. The mutant host cell strain of claim 1, wherein said mutation in said *rec*A gene is Δ(*srl-rec*A)1398.

7. The mutant host cell of claim 4, wherein said genetic element is a tetracycline resistance gene or transposon *Tn*10.

8. The mutant host cell of claim 1, wherein said host cell is an *Escherichia coli* cell.

9. The mutant host cell of claim 1, wherein said toxic gene is selected from the group consisting of *ccd*B, *kic*B, *Dpn*I, an apoptosis-related gene, a retroviral gene, a defensin, a bacteriophage lytic gene, ΦX *E*, an antibiotic

sensitivity gene, an antimicrobial sensitivity gene, a plasmid killer gene, and a eukaryotic transcriptional vector gene that produces a gene product toxic to bacteria.

10. The mutant host cell of claim 1, wherein said toxic gene is *ccd*B.

11. The mutant host cell of claim 1, wherein said host cell is selected from the group consisting of a DB3 cell (deposit number NRRL B-30097), a DB3.1 cell (deposit number NRRL B-30098), a DB4 cell (deposit number NRRL B-30106), and a DB5 cell (deposit number NRRL B-30107), or a mutant or derivative thereof.

12. Mutant host cell strain DB3 (deposit number NRRL B-30097).

13. Mutant host cell strain DB3.1 (deposit number NRRL B-30098).

14. Mutant host cell strain DB4 (deposit number NRRL B-30106).

15. Mutant host cell strain DB5 (deposit number NRRL B-30107).

16. A method of cloning a genetic construct comprising one or more toxic genes, said method comprising introducing said genetic construct into the host cell of claim 1 or claim 2 and cultivating said host cell under conditions favoring the clonal expansion of said host cell.

17. The method of claim 16, wherein said toxic gene is selected from the group consisting of *ccd*B, *kic*B, *Dpn*I, an apoptosis-related gene, a retroviral gene, a defensin, a bacteriophage lytic gene, ΦX *E*, an antibiotic sensitivity gene, an antimicrobial sensitivity gene, a plasmid killer gene, and a eukaryotic transcriptional vector gene that produces a gene product toxic to bacteria.

18. The method of claim 16, wherein said toxic gene is *ccd*B.

19. The method of claim 15, wherein said host cell is selected from the group consisting of a DB3 cell (deposit number NRRL B-30097), a DB3.1 cell (deposit number NRRL B-30098), a DB4 cell (deposit number NRRL B-30106), and a DB5 cell (deposit number NRRL B-30107), or a mutant or derivative thereof.

20. A kit comprising one or more of the mutant host cells of claim 1 or claim 2.

21. The kit of claim 20, further comprising one or more additional components selected from the group consisting of one or more culture media suitable for cultivation of said host cell, one or more selection agents, one or more genetic constructs comprising one or more toxic genes, one or more enzymes, one or more nucleotides, one or more buffers, and the like.

22. The kit of claim 19, wherein said host cell is selected from the group consisting of a DB3 cell (deposit number NRRL B-30097), a DB3.1 cell (deposit number NRRL B-30098), a DB4 cell (deposit number NRRL B-30106), and a DB5 cell (deposit number NRRL B-30107), or a mutant or derivative thereof.

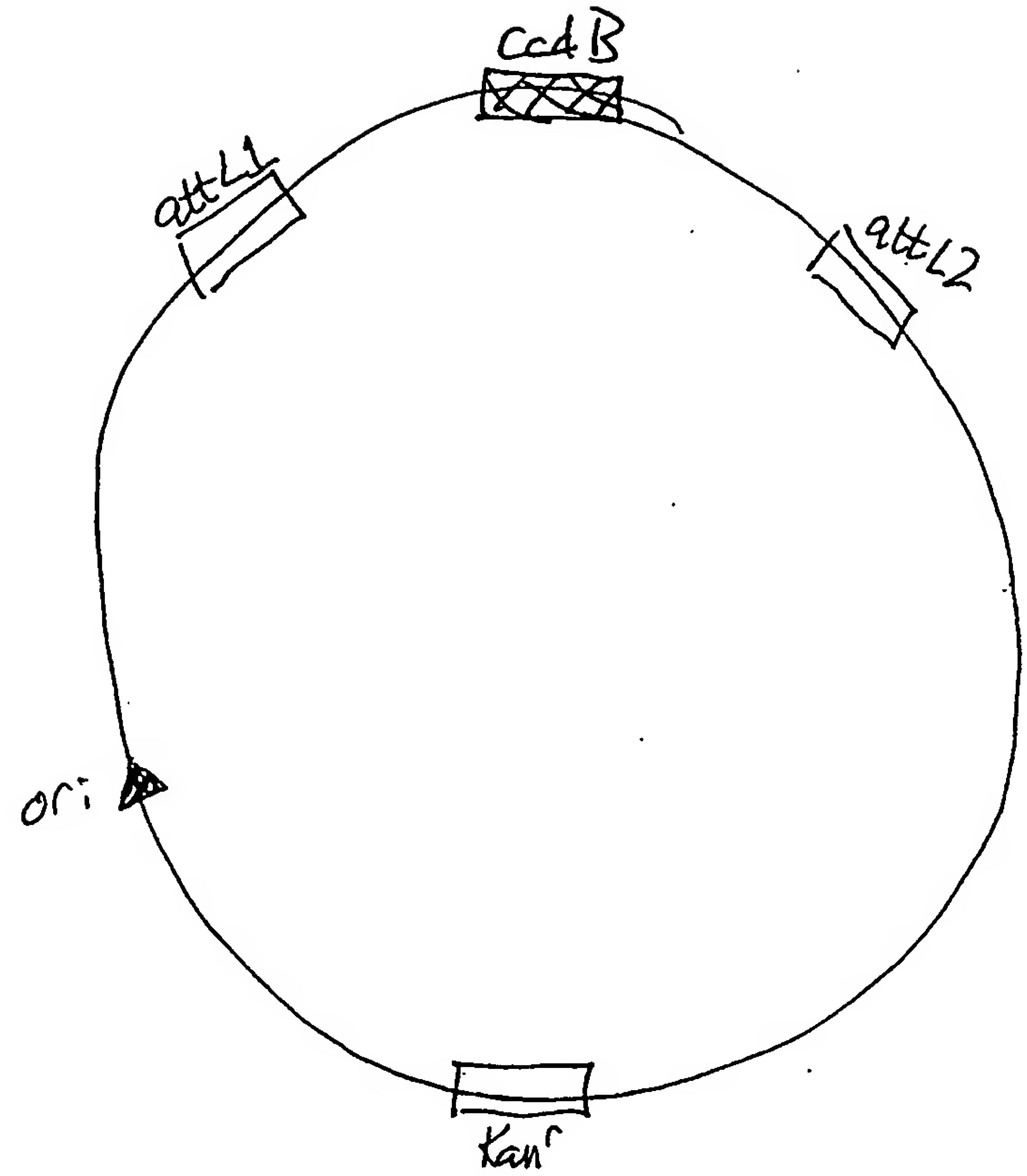


FIGURE 1

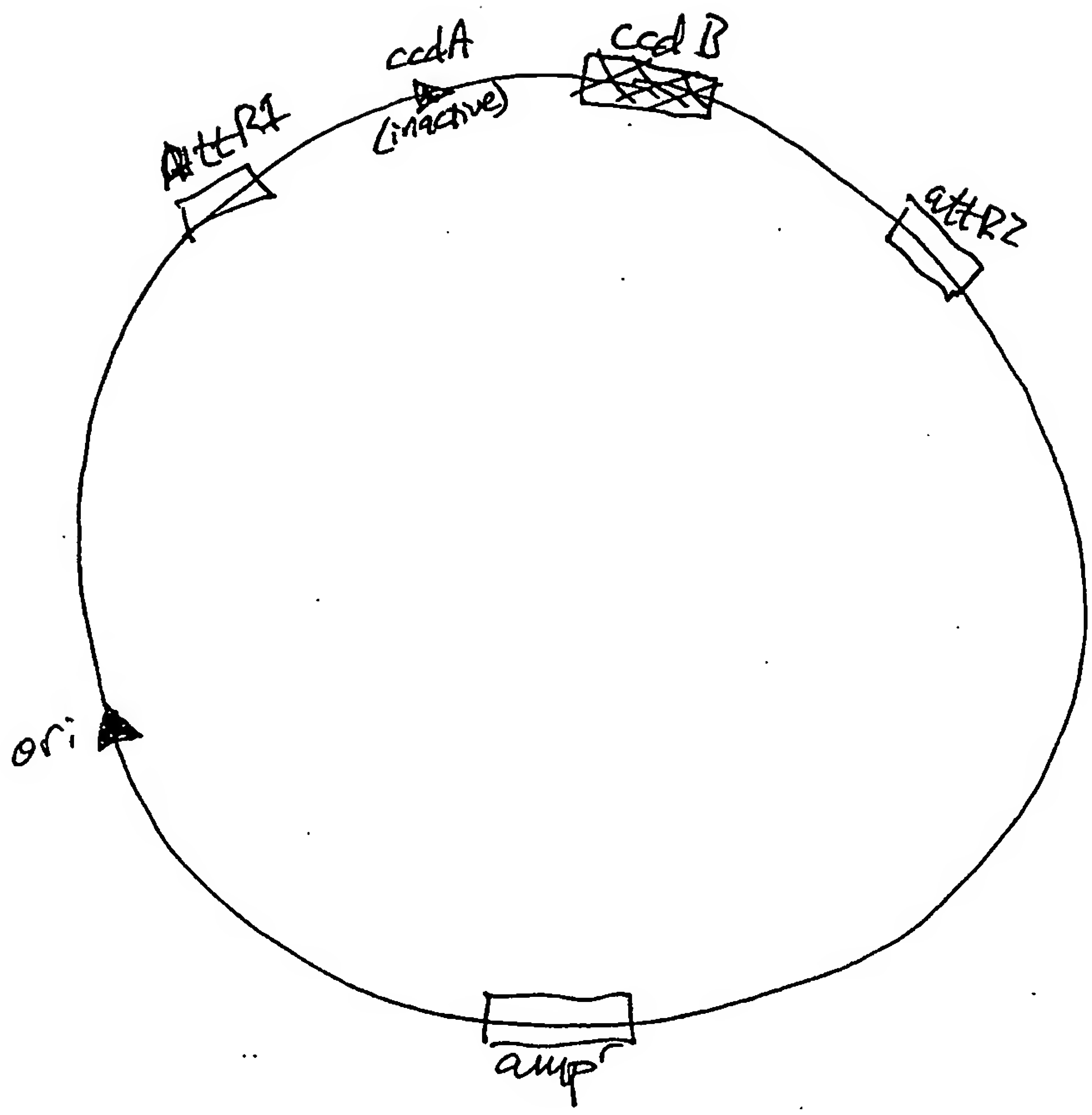


FIGURE 2

Figure 3 Cloning sites of the Entry Vector pENTR-7

attL1

```
--- ttg tac aaa aaa gca ggc ttt gaa aac ctg tat ttt caa gga
--- aac atg ttt ttt cgt ccg aaa ctt ttg gac ata aaa gtt cct
```

Leu Tyr Lys Lys Ala Gly Phe Glu Asn Leu Tyr Phe Gln Gly

TEV Protease

Xmn I Sal I Bam KpnI Eco RI

```
acc gtt tca tgc atc gtc gac tgg atc cgg tac cga att cgc ---
tgg caa agt acg tag cag ctg acc tag gcc atg gct taa gcg ---
```

Thr Val Ser Cys Ile Val Asp Trp Ile Arg Tyr Arg Ile

EcoR I Not I Xho I EcoR V Xba I

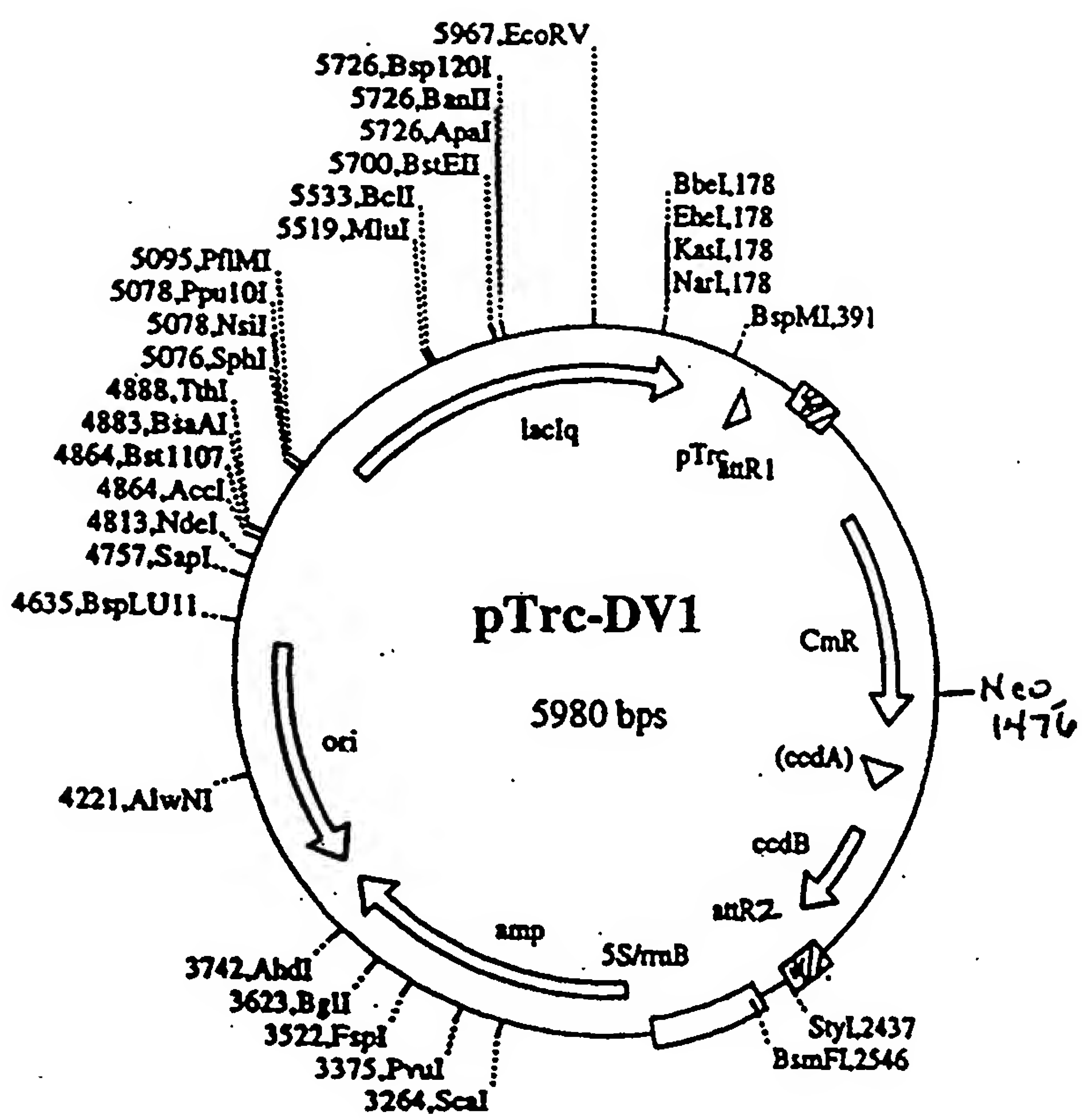
```
Death
(ccdB)   --- aga att cgc ggc cgc act cga gat atc tag acc cag
         --- tct taa gcg ccg gcg tga gct cta tag atc tgg gtc
```

Int

att L2

```
ctt tct tgt aca aag ---
gaa aga aca tgt ttc ---
```

FIGURE 4


5967,EcoRV
5726,Bsp120I
5726,BanII
5726,ApaI
5700,BstEII
5533,BclI
5519,MluI
5095,PflMI
5078,Ppu10I
5078,NsiI
5076,SphI
4888,TthI
4883,BsaAI
4864,Bst1107
4864,AccI
4813,NdeI
4757,SapI
4635,BspLU11
4221,AlwNI
3742,AhdI
3623,BglI
3522,FspI
3375,PvuI
3264,ScaI
BbeI,178
EheI,178
KasI,178
NarI,178
BspMI,391
lacIq
pTrc
attR1
CmR
(ccdA)
ccdB
attR2
5S/rrnB
amp
ori
StyI,2437
BsmFI,2546
Neo 1476
pTrc-DV1
5980 bps

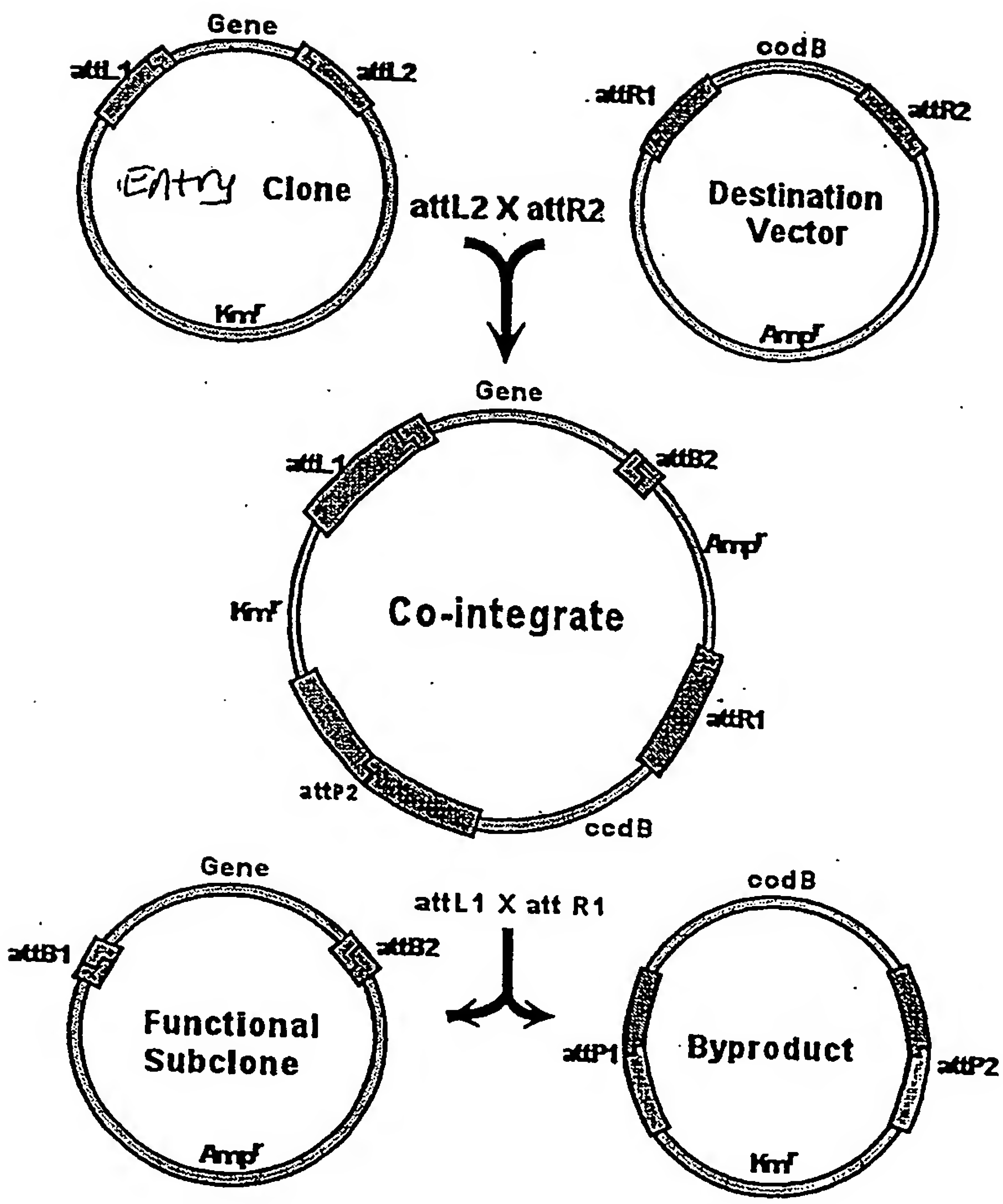

Gene
attL1
attL2
Entry Clone
KmR
attL2 X attR2
codB
attR1
attR2
Destination Vector
AmpR
Gene
attL1
attB2
AmpR
KmR
Co-integrate
attR1
attP2
ccdB
Gene
attB1
attB2
Functional Subclone
AmpR
attL1 X att R1
codB
attP1
Byproduct
attP2
KmR

FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/05246

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 1/21, 15/66
US CL :435/91.1, 252.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.1, 252.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BRS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,552,314 A (GREENER et al.) 03 September 1996(03.09.96), col. 3, lines 6-10.	1-22
X	US 5,695,971 A (KADOKAMI et al.) 09 December 1997(09.12.97), col. 13, lines 49-54.	1-22
Y,P	US 5,910,438 A (BERNARD et al.) 08 June 1999(08.06.99), col. 4, lines 12-17.	1-22
Y	BERNARD. P. et al. Positive-selection vectors using the F plasmid ccdB killer gene. Gene. 11 October 1994. Vol 148. No. 1. pages 71-74, especially page 73, Table I.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 APRIL 2000

Date of mailing of the international search report

24 JUL 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
JAMES KETTER
Telephone No. (703) 308-0196

WO0125466

Publication Title:

PRODUCER CELL FOR THE PRODUCTION OF RETROVIRAL VECTORS

Abstract:

A method is provided for modifying a producer cell which producer cell comprises integrated into its genome a provirus which provirus comprises one or more recombinase recognition sequences within or upstream of its 3' LTR, the method comprising: introducing into the cell a construct comprising a 5' recombinase recognition sequence, an LTR and a 3' recombinase recognition sequence in that order, in the presence of a recombinase which is capable of acting on the recombinase recognition site(s) such that the nucleotide sequence between the 5' and 3' recombinase recognition sequences in the construct is introduced into the provirus.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/25466 A1

(51) International Patent Classification[7]: C12N 15/867, 15/90, 5/10, 7/01, A61K 48/00

(21) International Application Number: PCT/GB00/03837

(22) International Filing Date: 5 October 2000 (05.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9923558.2 5 October 1999 (05.10.1999) GB

(71) Applicant (for all designated States except US): OXFORD BIOMEDICA (UK) LIMITED [GB/GB]; Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): SLINGSBY, Jason [GB/GB]; 91 Lacy Road, Putney, London SW15 1NR (GB). KINGSMAN, Susan, Mary [GB/GB]; Oxford BioMedica (UK) Limited, Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB). ROHLL, Jonathan [GB/GB]; Oxford BioMedica (UK) Limited, Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB). SLADE, Andrew [GB/GB]; Oxford BioMedica (UK) Limited, Medawar Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA (GB).

(74) Agents: HARDING, Charles, Thomas et al.; D Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PRODUCER CELL FOR THE PRODUCTION OF RETROVIRAL VECTORS

(57) Abstract: A method is provided for modifying a producer cell which producer cell comprises integrated into its genome a provirus which provirus comprises one or more recombinase recognition sequences within or upstream of its 3' LTR, the method comprising: introducing into the cell a construct comprising a 5' recombinase recognition sequence, an LTR and a 3' recombinase recognition sequence in that order, in the presence of a recombinase which is capable of acting on the recombinase recognition site(s) such that the nucleotide sequence between the 5' and 3' recombinase recognition sequences in the construct is introduced into the provirus.

WO 01/25466 A1

PRODUCER CELL FOR THE PRODUCTION OF RETROVIRAL VECTORS

FIELD OF THE INVENTION

The present invention relates to retroviral vectors, in particular to high titre regulatable retroviral vectors.

BACKGROUND TO THE INVENTION

Retroviruses have been proposed as a delivery system (otherwise expressed as a delivery vehicle or delivery vector) for *inter alia* the transfer of a nucleotide sequence of interest (NOI), or a plurality of NOIs, to one or more sites of interest. The transfer can occur *in vitro*, *ex vivo*, *in vivo*, or combinations thereof. When used in this fashion, the retroviruses are typically called retroviral vectors or recombinant retroviral vectors. Retroviral vectors have been exploited to study various aspects of the retrovirus life cycle, including receptor usage, reverse transcription and RNA packaging (reviewed by Miller, 1992 Curr Top Microbiol Immunol 158:1-24).

In a typical recombinant retroviral vector for use in gene therapy, at least part of one or more of the *gag, pol* and *env* protein coding regions may be removed from the virus. This makes the retroviral vector replication-defective. The removed portions may even be replaced by a NOI in order to generate a virus capable of integrating its genome into a host genome but wherein the modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic effect. Thus, the transfer of a NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a virion coat; and allowing transduction of a site of interest - such as a targetted cell or a targetted cell population.

It is possible to propagate and isolate quantities of retroviral vectors (e.g. to prepare suitable titres of the retroviral vector) for subsequent transduction of, for example, a site of interest by using a combination of a packaging or helper cell line and a recombinant vector.

In some instances, propagation and isolation may entail isolation of the retroviral *gag*, *pol* and *env* genes and their separate introduction into a host cell to produce a "packaging cell line". The packaging cell line produces the proteins required for packaging retroviral RNA but it does not produce RNA-containing retroviral vectors. However, when a recombinant vector carrying a NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This can be used to infect cells to introduce the NOI into the genome of the cells. The recombinant virus whose genome lacks all genes required to make viral proteins can infect only once and cannot propagate. Hence, the NOI is introduced into the host cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

Retroviral packaging cell lines have been developed to produce retroviral vectors. These cell lines are designed to express three components, which may be located on three separate expression constructs. The *gag/pol* expression construct encodes structural and enzymatic components required in particle formation, maturation, reverse transcription and integration. The envelope (*env*) construct expresses a retroviral or non-retroviral envelope protein, which mediates viral entry into cells by binding to its cognate receptor. The third expression construct produces the retroviral RNA genome containing a *psi* region, which is packaged into mature, enveloped retroviral particles.

It has been observed that different methods, such as electroporation, transfection and retroviral transduction, which have been used to introduce the retroviral expression construct for the RNA genome, termed "the genome", into packaging cells produce different results. These different results can include different end points or "yield" of retroviral producer lines resulting from the derived cell lines. Moreover, electroporation and transfection methods can be problematic in the sense that the titre levels are not always at a satisfactory level.

By way of example, the transfection of a plasmid DNA construct into packaging cells from a MLV packaging cell line of human origin, called FLYA13, yielded low retroviral

vector titres even when different transfection reagents such as calcium phosphate precipitation and fugene transfection reagent were used. The average titres from selected stably transfected cell lines clones ranged from about 10^3 to about 10^4 per ml. In addition, clones generated by electroporation of constructs gave similar titres of from about 10^3 to about 10^4 per ml with no clones identified producing at $>10^5$ per ml. However, when MLV vector particles are prepared in a transient transfection system with a different envelope pseudotype to the packaging cell, and used to transduce a retroviral packaging cell, stably transduced cell lines made by this transduction method produce retrovirus at 10^6 to 10^7 per ml. Therefore, these results suggest that retroviral transduction is a preferred method for genome introduction into packaging cell lines in order to generate high titre producer cell lines. However, when retroviral transduction is used to introduce a regulated/inactivated retroviral vector genome into packaging cell lines, the regulated retroviral vectors may not be produced in sufficient quantities from these cell lines.

By way of example, some retroviral vectors may comprise (i) internal expression constructs which are themselves regulated or (ii) regulated elements which are present in retroviral 3' LTR sequences, either by design or by their nature. Examples of these regulated vectors include but are not limited to hypoxic regulated vectors and self inactivating (SIN) vectors. If transduced producer cell lines are generated with these regulated vectors, the regulated or inactivated 3' U3 sequence of the LTR is copied to the 5' LTR by the process of retroviral reverse transcription and integration. Therefore, in the producer cell line, the 5' U3 promoter sequence directing expression of retroviral RNA genomes is identical to the regulated or inactivated 3' U3 promoter. This will result in very low levels of retroviral genome production and consequently low titres of functional retrovirus vectors being produced.

One example of such a regulated retroviral system includes MLV and lentivirus vector constructs where the 3' retroviral U3 enhancer element is replaced with a hypoxia responsive element (HRE) or other physiologically regulated, tumour specific or tissue-specific promoters. When these vectors are used to make a transduced producer cell line, the 3' U3 sequence containing the HRE element is copied to the 5' LTR position and retroviral genomes will only be produced under hypoxic conditions or chemical mimics